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Prevalence of Plasmid-Mediated Quinolone Resistance (PMQR) Determinants Among Extended Spectrum Beta-Lactamases (ESBL)-Producing Isolates of *Escherichia coli* and *Klebsiella pneumoniae* in Aleppo, Syria

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Background: Recently, several plasmid-mediated quinolone resistance (PMQR) genes conferring low levels of quinolone resistance have been discovered.
Objectives: The aim of the present study is to determine the prevalence of plasmid-mediated quinolone resistance genes in a collection of ESBL-producing isolates of *E. coli* and *K. pneumonia* in Aleppo, Syria.
Materials and Methods: Here, to evaluate the prevalence of PMQR genes at Aleppo University hospitals in Syria, 123 extended spectrum beta-lactamases (ESBL)-producing isolates of *Escherichia coli* and *Klebsiella pneumoniae* from the hospitals were selected for screening based on ciprofloxacin resistance. Five PMQR genes [*qnrA*, *qnrB*, *qnrS*, *aac (6')-Ib*, and *qepA*] were screened by simplex PCR, and the *aac (6')-Ib*-positive PCR products were digested with *Bts*CI to detect the *aac (6')-Ib-cr* variant.
Results: Of the 123 isolates, 103 (83.73%) had one of the five PMQR genes, including 83 (83.83%) of the 99 *E. coli* strains and 20 (86.95%) of the 23 *K. pneumoniae* strains.
Conclusions: The most common *qnr* gene was *qnrB*, and none of the isolates carried *qnrA* or *qepA*. The *aac (6')-Ib-cr* variant was the most prevalent PMQR gene, and it was associated with the prevalence of ciprofloxacin resistance in our ESBL-producing isolates.

Keywords: QepA Protein; Beta-Lactamase; Ciprofloxacin; QepA Protein; *Escherichia coli*; *Klebsiella pneumoniae*

1. Background

Quinolones and fluoroquinolones are broad-spectrum antimicrobial agents that are used extensively in both medical and veterinary practice. Therefore, residues from these antibiotics are present in the environment (1). This widespread use is associated with an increased level of quinolone resistance, particularly within the last 10 years (2). Bacterial resistance to quinolones commonly results from chromosomal mutations (3, 4). However, several recent studies showed that quinolone resistance can also be mediated by plasmid-carried genes, so called plasmid-mediated quinolone resistance (PMQR) genes (2), which encode the Qnr proteins, which protect the quinolone targets (5); the Aac (6')-Ib-cr enzyme, which acetylates not only aminoglycosides but also ciprofloxacin and norfloxacin (5); and QepA, a plasmid-encoded efflux pump (5).

The first plasmid-mediated quinolone resistance gene, named *qnr,* was reported in 1998. This gene encodes a 218-amino acid protein, which was later renamed QnrA; it is a member of the pentapeptide-repeat family. More recently, four additional proteins, QnrB, QnrS, QnrC, and QnrD, have been identified in several enterobacterial species (6, 7). These proteins interact with quinolones, topoisomerases, and DNA, and act by limiting the binding of quinolones to their targets (8). By itself, the *qnr* gene confers low-level resistance to quinolones. However, the presence of this gene facilitates the acquisition of high-level resistance among initially susceptible strains (9, 10).

In 2005, a second plasmid-mediated mechanism that independently contributes to quinolone resistance through modification of the antibiotic molecule was described; the encoded protein, Aac (6')-Ib-cr, is a variant of 6'acetyl transferase, which is known to modify the chemical structure of aminoglycosides, and it confers broad spectrum resistance to ciprofloxacin and norfloxacin (11).

The third PMQR mechanism is mediated by QepA, an efflux pump belonging to the major facilitator subfamily, which pumps fluoroquinolones out of bacterial cells (12, 13).

PMQR determinants have been identified worldwide, with varying prevalence rates (9). Their presence is asso-

ciated with resistance to other antimicrobial agents, particularly to β-lactams (2).

In Aleppo, there have been no studies published on the incidence of ciprofloxacin resistance and its association with PMQR genes.

2. Objectives

The aim of the current study is to determine the prevalence of plasmid-mediated quinolone resistance genes in a collection of ESBL-producing isolates of *E. coli* and *K. pneumonia* in Aleppo, Syria.

3. Materials and Methods

3.1. Bacterial Isolates

A total of 123 non-duplicate ESBL-producing isolates (99 *E. coli* and 24 *K. pneumoniae* isolates) were obtained from three university hospitals offering tertiary medical care in Aleppo city between October 2010 and June 2011 (14). These isolates were stored in microvials (Microbank™) at -80°C.

3.2. Antimicrobial Susceptibility Testing

A broth microdilution method was used to determine the minimum inhibitory concentration (MIC), in accordance with the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) guidelines (15). Each bacterial suspension was adjusted to 1 McFarland standard, which contains approximately 1.5×10^8 cfu/mL, and then diluted with broth to a final density of 5×10^5 cfu/well, and inoculated onto microplates containing the test drug at various concentrations. Each plate was incubated at 35 - 37°C for 18 hours. The reference strains *E. coli* ATCC25922 and *K. pneumoniae* ATCC700603 were included in each run as controls. The MIC breakpoints used for susceptibility and resistance to ciprofloxacin were ≤1 μg/mL and ≥4 μg/mL, respectively.

3.3. Screening Procedures

Plasmids were extracted using the QIAprep Miniprep Kit (Qiagen), according to the manufacturer's instructions. Screening for *qnrA*, *qnrB*, *qnrS*, *aac (6')-Ib*, and *qepA* was performed by simplex polymerase chain reaction (PCR) with specific primers and PCR conditions that have been previously described (16-19).

Negative controls (without DNA template) were included in each run. Amplification products were identified by their sizes on 1.5% agarose gels after electrophoresis at 130 V for 25 min and staining with ethidium bromide.

All PCR products positive for *aac (6')-Ib* were further analyzed by digestion with *Bts*CI (Thermo scientific) to identify *aac (6')-Ib-cr*, which lacks the *Bts*CI restriction site that is present in the wild-type gene (16). The wild-type *aac (6')-Ib* PCR product yielded 210-bp and 272-bp fragments after restriction (18). Fragments were extracted from the gel by using the QIA prep Miniprep Kit (Qiagen).

4. Results

4.1. Phenotype Confirmation

The MIC of ciprofloxacin ranged from < 0.125 μg/mL to > 128 μg/mL. We found that 65.81% of tested isolates were resistant to ciprofloxacin. The MICs of the tested antimicrobial agents for the isolates are shown in Table 1.

4.2. Prevalence of PMQR Genes

In total, 123 isolates were included in this study. Only 34.14% (42/123) were confirmed to have at least one of the three *qnr* genes. The prevalence of each PMQR gene is shown in Table 2.

Qnr genes were detected more frequently in *K. pneumoniae* (62.5%, 15/24) than in *E. coli* (27.27%, 27/99). Both *qnrB* and *qnrS* were significantly more prevalent in *K. pneumoniae* isolates than in *E. coli* isolates. *Aac (6')-Ib-cr* was the most prevalent PMQR gene in our isolate pool (75.6%, 93/123), and *aac (6')-Ib-cr* accounted for 94% (93/98) of all *aac (6')-Ib* genes detected. Neither *qnrA* nor *qepA* was detected.

The distribution of PMQR determinants among the tested isolates and their corresponding MICs are shown in Table 3.

Table 1. Distribution of the Minimum Inhibitory Concentrations of Ciprofloxacin for *Escherichia coli* and *Klebsiella pneumoniae* Isolates

MIC (μg/mL) [a]	Isolates [b]
Sensitive isolates	
< 1/8	18 (14.63)
1/8	2 (1.62)
1/4	6 (4.87)
1/2	3 (2.43)
1	1 (0.81)
Intermediate isolates	
2	12 (9.75)
Resistant isolates	
4	12 (9.75)
8	2 (1.62)
16	5 (4.06)
32	12 (9.75)
64	25 (20.32)
128	6 (4.87)
> 128	19 (15.44)

[a] MIC, minimum inhibitory concentration.
[b] Values are presented as No. (%).

Table 2. Prevalence of the Five Plasmid-Mediated Quinolone Resistance Determinants [a]

Species	Total No. of Isolates	No. of Positive Isolates for PMQR Genes					
		qnrA	*qnrB*	*qnrS*	*aac (6')-Ib*	*aacv (6')-Ib-cr*	*qepA*
Escherichia coli	99 (80.5)	0	18 (18.1)	11 (11.1)	80 (80.8)	78 (78.78)	0
Klebsiella pneumoniae	24 (19.5)	0	12 (50)	9 (37.5)	18 (75)	15 (62.5)	0
Total	123	0	30 (24.4)	20 (16.3)	98 (79.7)	93 (75.6)	0

[a] Values are presented as No. (%).

Table 3. Minimum Inhibitory Concentration (MIC) Values of Plasmid-Mediated Quinolone Resistance-Positive Isolates

Genes	Isolates (n)	MIC μg/mL (%)			
		< 1/8 - 1/2	1 - 4	8 - 32	64 - 128 <
qnrB					
Escherichia coli	18	4 (22.2)	2 (11.1)	3 (16.6)	9 (50)
Klebsiella pneumoniae	12	2 (16.6)	3 (25)	3 (25)	4 (33.3)
qnrS					
Escherichia coli	11	1 (9)	2 (18.1)	1 (9)	7 (63.6)
Klebsiella pneumoniae	9	1 (11.1)	3 (33.3)	0	5 (55.5)
aac (6')-Ib-cr					
Escherichia coli	78	18 (23)	13 16.6)	14 (17.9)	33 (42.3)
Klebsiella pneumoniae	15	1 (6.6)	3 (20)	2 (13.3)	9 (60)
qnrB* + *qnrS					
Escherichia coli	2	0	0	1 (50)	1 (50)
Klebsiella pneumoniae	6	1 (16.6)	2 (33.3)	0	3 (50)
qnrB* + *aac (6')-Ib-cr					
Escherichia coli	15	3 (20)	2 (13.3)	3 (20)	7 (46.6)
Klebsiella pneumoniae	8	0	2 (25)	2 (25)	4 (50)
qnrS* + *aac (6')-Ib-cr					
Escherichia coli	9	1 (11.1)	1 (11.1)	1 (11.1)	6 (66.6)
Klebsiella pneumoniae	6	0	2 (33.3)	0	4 (66.6)
qnrB* + *qnrS* + *aac (6')-Ib-cr					
Escherichia coli	2	0	0	1 (50)	1 (50)
Klebsiella pneumoniae	4	0	1 (25)	0	3 (75)

5. Discussion

It has been more than 30 years since fluoroquinolones were first introduced in Syria. A Syrian study suggested that the resistance rate against ciprofloxacin has reached 39.1%, with upward trends in the use of fluoroquinolones in community and hospital settings (14). In the present study, 65.81% of the ESBL-producing isolates from Aleppo University Hospitals were resistant to ciprofloxacin. Therefore, we investigated the prevalence of PMQR determinants and analyzed their association with phenotypic ciprofloxacin resistance.

Previous studies reported that *qnr* genes were rare (20); however, in the present study, we found that the prevalence of *qnr* genes in our study was higher (34.14%) than that reported in other studies (21-23). Although the prevalence of each PMQR gene varied by species, in general, *qnr* genes were more prevalent in *K. pneumoniae* (62.50%, 15/24) than in *E. coli* (27.27%, 27/99), as was previously described in studies conducted in France (24), the United States (17), Spain (21), and China (25). The most frequently detected *qnr* gene was *qnrB* (24.4%), as has been reported in in other studies (23, 26), and we also noted an absence of *qnrA*, which has also been reported previously (1, 18, 23, 26-28).

Notably, there was no statistically significant association between *qnr* and ciprofloxacin resistance, and *qnr* genes were common among both ciprofloxacin-sensitive/intermediate isolates (28.5%, 12/42) and resistant isolates (37%, 30/81). Our findings agree with other reports demon-

strating that *qnr* alone does not confer resistance to fluoroquinolones. However, its presence may facilitate the selection of additional chromosomal mechanisms, such as changes in DNA gyrase (*gyrA*) and/or topoisomerase IV (*parC*) genes (2, 10, 17, 26, 29), and the presence of *qnr* does not necessarily lead to MICs above the CLSI breakpoints for resistance to ciprofloxacin (30). Furthermore, using ciprofloxacin breakpoints as markers for detection may underestimate the prevalence of *qnr* genes, which raises concern for the undetected spread of these genes (23). Consequently, infections caused by *qnr*-positive isolates might be treated with quinolones, thus enhancing the selection of resistant mutants (2) and increasing the risk of therapeutic failure (23).

We noted the absence of *qepA* among the studied strains. The QepA efflux pump, first described in 2007 in two *E. coli* clinical isolates from Japan and Belgium (12, 13), has already been detected in France, with a new variant QepA2 (31). However, *qepA* is still very rare, except in China where two recent studies underlined the predominance of the *qepA* gene in enterobacterial strains isolated from food-producing animals. The most surprising finding of our study was the wide penetration of the *aac (6')-Ib-cr* allele, which was more prevalent (75.6%) than the *qnr* genes (43.14%). Notably, *aac (6')-Ib-cr* accounted for 94% (93/98) of the *aac (6')-Ib* genes detected.

This high proportion of *aac (6')-Ib-cr|aac (6')-Ib* has also been reported in other studies (11, 25), and it probably reflects an extended emergence and ongoing dissemination of under detected *aac (6')-Ib-cr*. Moreover, its presence as part of an integron cassette (11, 32) suggests that it could be widely mobile among plasmids.

Although the *qnr* genes were predominant in *K. pneumoniae*, *aac (6')-Ib-cr* was the most prevalent PMQR gene in *E. coli* (78/99, 63.4%), and it was much less prevalent in *K. pneumonia* (12.20%, 15/24). These differences are in agreement with previous observations (16, 18, 23); however, the reason for these differences is not yet understood, since it is known that some plasmids can carry both *aac (6')-Ib-cr* and *qnrA* genes (17).

To investigate the contribution of the *aac (6')-Ib-cr* gene to ciprofloxacin resistance, we analyzed the relationship between the presence of *aac (6')-Ib-cr* and resistance to ciprofloxacin. Our resistant isolates were significantly more frequently *aac (6')-Ib-cr*–positive (81.8%, 66/81) than our sensitive/intermediate isolates (64.28%, 27/42). Thus, *aac (6')-Ib-cr* was significantly associated with phenotypic ciprofloxacin resistance (P = 0.02).

There was no relationship between the presence of *qnrA*, *qnrB*, or *qnrS* and *aac (6')-Ib-cr*; *qnr* genes were present in 33.33% (31/93) of the *aac (6')-Ib-cr*-positive strains and 28.57% (10/35) of the *aac (6')-Ib-cr*-negative strains, indicating that the *qnr* genes and *aac (6')-Ib-cr* can circulate independently. This result is consistent with some previous results (16), but is in contrast to other results from China where the *aac (6')-Ib-cr* variant was detected in 55.2% of *qnr*-positive in *E. coli* and *K. pneumoniae* isolates but in only 6% of *qnr*-negative isolates (25). In conclusion, our study showed that the prevalence of plasmid-mediated *qnr* and *aac (6')-Ib-cr* quinolone resistance genes was high among Syrian clinical ESBL-producing isolates of *E. coli* and *K. pneumonia*, and that this association should be further studied in the future. However, the distribution of the *aac (6')-Ib-cr* variant differed between the two species; it was detected more often in *E. coli* isolates than in *K. pneumonia* isolates, which is the reverse of that for *qnr* genes. Conversely, the *qnr* genes were more prevalent in *K. pneumoniae* than in *E. coli*, and *qnrB* was more prevalent than *qnrA* or *qnrS*, and the prevalence of ciprofloxacin resistance in our isolates was associated with the prevalence of the *aac (6')-Ib-cr* variant.

Finally, it seems likely that the increasing use of fluoroquinolones over the last 10 years created an opportunity for the emergence of ciprofloxacin-resistant clinical isolates with PMQR determinants. Additional regional epidemiological data on antimicrobial resistance throughout Syria is needed to promote appropriate antimicrobial therapy and effective infection control. In addition, ensuring the use of antibiotics that are not substrates for *aac (6')-Ib-cr* might reduce selection pressure for this variant but not for the *qnr* genes.

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Authors' Contributions

Study concept and design: Rawaa Al kayali; acquisition of data: Omar Alheib; analysis and interpretation of data: Rawaa Al kayali; drafting of the manuscript: Omar Alheib; critical revision of the manuscript for important intellectual content: M. Yaser Abajy; statistical analysis: Rawaa Al kayali and Omar Alheib; administrative, technical, and material support: M. Yaser Abajy and Omar Alheib; study supervision: Rawaa Al kayali.

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